

The Role of Fucose in Early Cancer Detection

INTRODUCTION

In many disease states, biological changes occur that indicate disease progression. For example, protein from cancer patients is commonly observed with an increased amount of the sugar fucose. Fucose levels are often elevated even before cancer symptoms are present; thus, fucose is an important indicator that a patient has cancer. The goal of this research is to develop a simple test for fucose levels to assist with early detection of cancer. This research involves preliminary tests to detect fucose at very low levels. These rapid methods could potentially be used in clinical tests for patients who are at risk. Two instrumental methods are also described, which provide sensitive and accurate quantitative results to verify the rapid methods.

BACKGROUND/METHODS

Diagnosing cancer in the early stages can be vital for improving patient treatment and survival rates. Significant research shows that biomarkers, or indicators of certain

biological states, can be used to determine when cancer is present or has metastasized.¹ Biomarkers like fucose are often detectable at the very early stages of cancer;² so investigating these biomarkers is a growing field that is beneficial to patients at all stages of cancer diagnosis and treatment. Thus, the goal of this project is to develop quick, simple methods for detecting and quantifying fucose. These biomarker detection techniques can hopefully be applied beyond the laboratory and in a clinical setting, where a quick and easy method to determine fucosylation is vital to cancer prognosis.

Proteins in the human body undergo numerous changes after they are produced in the cells. Glycosylation, or the addition of sugars to proteins, is ubiquitous in human proteins. Protein glycosylation is important for regulating cellular signaling, cell differentiation and has proven to be a biomarker for certain disease states.^{3,4} Changes in glycosylation occur in a variety of diseases, ranging from cancer to cystic fibrosis.⁵ For

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example, elevated fucose levels on glycoproteins is linked to human hepatocellular carcinoma and even breast cancer.^{6,7} Measuring changes in fucosylation is a vital diagnostic tool for early cancer detection; changes in sugars often occur before cancer has fully developed.⁸

Four analytical techniques were utilized to study and quantify fucose. The group investigated two common color-change reactions for detecting sugars. Colorimetric indicators are useful because the amount of sugar in a sample can easily be visualized without extra equipment. The first rapid method uses phenol and sulfuric acid. When added to a sugar solution, these compounds create a yellow dye; the shade of yellow depends upon the sugar concentration. This reaction was central to the Yellow Card method, in which a yellow scale was designed for visual determination of fucose concentration. Potassium permanganate also reacts with reducing sugars to produce a color change that indicates fucose concentration. This Colorized Reducing Sugar (CRS) test was the second simple method. Two instrumental techniques were also chosen to accurately quantify fucose: High Performance Liquid Chromatography with Pulsed Amperometric Detection and Liquid Chromatography with Mass Spectrometry.

It is necessary to study fucose at concentrations that are biologically relevant to ensure that experiments in the lab would be viable as clinical tests. Although these studies work with pure fucose rather than blood or serum samples, the fucose solutions were prepared based on characteristic fucose levels in the body. The group designated three classes based on fucose levels typical of oral cancer development. A normal, or cancer-free, individual would have fucose levels at 800 μM ; precancerous levels

are at 1200 μM ; the threshold for cancer is at 1600 μM .⁹ Experiments were designed using this classification system.

All methods were tested in the range stated above for preliminary experiments. Two test samples of fucose were also analyzed by each method. One group member prepared fucose solutions at concentrations of 933 μM and 1314 μM . These samples were regarded as “unknown,” as the individuals testing these samples did not know their concentrations. The “unknown” samples were analyzed with each method. Experimental design is described below.

RAPID METHODS

The simple Yellow Card method is based on the colorization of sugar solutions upon addition of phenol and sulfuric acid. In the reaction, each monosaccharide molecule is dehydrated by the acid, forming a furfural derivative. The furfural derivative then condenses with two molecules of phenol to form a yellow dye molecule. The result is that one molecule of yellow dye is formed for each molecule of sugar in the solution. More concentrated sugar solutions, therefore, produce more intense yellow coloring.

The color change occurs in all carbohydrate solutions, and only a small amount of fucose was available for experimentation, so experiments were performed with other sugars before testing methods on fucose solutions. Galactose is very similar to fucose, so it was chosen for the initial colorization experiments. Galactose solutions in a linear range of concentrations (200 μM to 2000 μM) were prepared and reacted with phenol and sulfuric acid. The resulting solutions ranged in color from nearly clear to golden yellow. The experiment was then performed on fucose solutions, producing a similar range

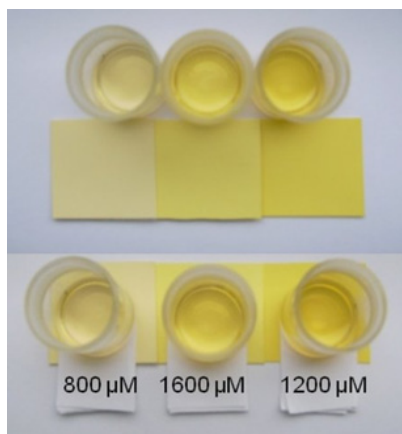


Figure 1. (left) Fucose solutions after treatment with phenol and sulfuric acid are placed on white backgrounds and can be seen to match the correct sections of the Yellow Card. The colors included in the tricolored care are, from left to right, Honey Pot (ACE), Yellow Brick Road (ACE), and Citrus (Behr).

Figure 2. (above) Fucose, 15μL Indicator, 800μM, 1200μM, 1600μM left to right, 30 min.

of yellow hues. The 800 μM, 1200 μM, and 1600 μM fucose solutions were visibly distinguishable, suggesting that this range of concentrations would be useful for visual distinction between normal and elevated serum fucosylation levels.

To develop the actual Yellow Card, fucose solutions of 800 μM, 1200 μM, and 1600 μM concentrations were reacted with phenol and sulfuric acid in identical vials, and their yellow hues were matched to color swatches from paint manufacturers. A pale yellow color matched the 800 μM solution and two darker shades of yellow matched the 1200 μM and 1600 μM solutions. These three colors are included in the tricolored “Yellow Card” and serve as references when estimating fucose concentrations (Figure 1).

The Colorized Reducing Sugar (CRS) test utilizes an oxidation-reduction reaction in which the sugar’s aldehyde group is oxidized to a carboxylic acid and permanganate is reduced to manganese. Permanganate is purple in solution, while manganese is colorless. Solutions are colorized by the permanganate indicator, but each molecule of sugar in the solution removes some of the purple color. The color of the resulting

solution, therefore, depends upon concentration of the sugar. The 800μM fucose solution produced a maroon color, 1200μM fucose solution produced a burnt-orange color, and 1600μM fucose solution produced a caramel-brown (Figure 2).

Multiple sugars, including glucose, mannose, and galactose were tested as model systems for fucose. These were analyzed in the same concentration range and the same method was used as was used with fucose. All of the sugars produced similar colors when treated with indicator, but the color change occurred more rapidly for some sugars. Mannose reacted the fastest: the 1600μM sample turned brown within five minutes, then glucose—producing the caramel color in 10 minutes, and galactose was slowest—it required 50 minutes for the 1600μM sample to yield the caramel color (Figures 3-5).

Variables such as concentration of sugar, volume of potassium permanganate, and time of heating were tested; it was determined that the concentration range that displays a color variation was from about 700μM to 1800μM (700μM being the lower limit of detection). Indicator (potassium permanganate)

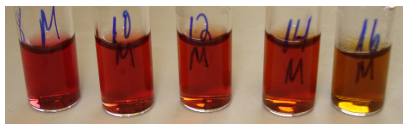


Figure 3. Mannose, 20 μ L Indicator, 800, 1000, 1200, 1400, 1600 μ M left to right, 5 min.

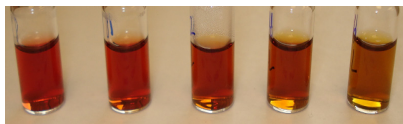


Figure 4. Glucose, 20 μ L Indicator, 800, 1000, 1200, 1400, 1600 μ M left to right, 10 min.

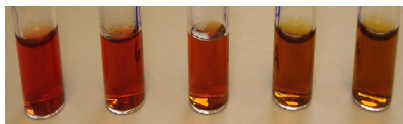


Figure 5. Galactose, 20 μ L Indicator, 800, 1000, 1200, 1400, 1600 μ M left to right, 50 min.

volume affected the color change slightly. Solution colors were more distinguishable and darker when higher volumes of indicator were applied (Figure 6). Heating time affected results greatly—reaction rate increased as time on the heat source increased. Optimal results were obtained when fucose solutions were heated for one minute on a 90°C heat source, then removed and immediately treated with the indicator. The color of the resulting solutions changed over time, and it was determined that different fucose concentrations were most distinguishable when observed 20-30 minutes after addition of the indicator. Because the color change occurs for any reducing sugar, only pure fucose solutions were utilized for quantitative experiments.

INSTRUMENTAL METHODS

Although the simple methods stated above are quick, they do not have the quantitative capability of an instrumental technique. Thus, to explore more quantitative techniques,



Figure 6. Fucose, 20 μ L Indicator, left to right: 800 μ M, 1200 μ M, 1600 μ M, 30 minute reaction time. A higher volume of indicator improved the reaction and made the three concentrations of fucose more visually distinguishable.

two instrumental methods were applied that provide sensitive and quantitative results.

High performance liquid chromatography with pulsed amperometric detection (HPLC-PAD) is a highly sensitive method for separating and detecting the components of a solution. This method utilizes an anion exchange column, separating fucose through interactions of its anionic forms with the substrate of the column. Different sugars have varying affinities for the column; thus move through it at different rates, and leave the column at specific times. Knowing these times for fucose allowed for identification and accurate quantitation.

Pulsed amperometric detection (PAD) was used with HPLC to detect fucose electrochemically. Amperometry was used with three electrodes to drive an electrochemical reaction. One of the electrodes is the working electrode, where the electrolysis takes place. The second electrode, the counter electrode, allows for the flow of current by acting as an “electron sink”. For work with pulsed amperometric detection, a third non-polarizable electrode, the reference electrode, was needed so that a comparison between the changes in current could be measured. The change in current corresponded to the concentration of fucose in a

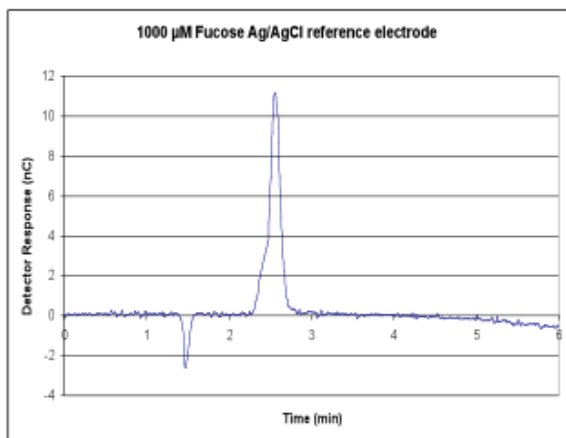


Figure 7. This HPLC-PAD chromatogram depicts 1000 μM fucose, which comes off the amino column at approximately 2.5 minutes to produce the peak.

sample. This method was applied to the samples run on the rapid colorimetric methods to confirm the accuracy of the results.

Quantitation by HPLC-PAD was done using a calibration curve as an external standard. Five fucose solutions (2000 μM , 1000 μM , 500 μM , 250 μM , and 125 μM) were made by serial dilution. Each solution was analyzed by HPLC-PAD three times, producing chromatograms (Figure 7). Literature relevant to the detection of simple carbohydrates using HPLC-PAD was consulted to select the optimal amperometric conditions. The fucose peak areas from the chromatograms were plotted versus concentration, and linear regression curve was made to fit the data. This produced a standard curve for the extrapolation of concentrations of unknown samples.

Along with HPLC-PAD, liquid chromatography-mass spectrometry (LC-MS) was used to verify the results of the rapid methods. With HPLC-PAD, separation occurs in the liquid phase inside a column. However, with LC-MS, compounds were separated by an amino column. Optimal separation

was produced by a mobile phase containing 60% ACN and 40% H_2O .

Mass spectrometry was used as to identify and quantify fucose after liquid chromatography. MS provides mass-specific information about compounds, which can be helpful for analysis. Utilizing electrospray ionization mass spectrometry (ESI-MS); samples were ionized

with 10 mM ammonium acetate in positive ion mode and detected by the spectrometer. This ionization produced the charge state $[\text{M}+\text{NH}_4]^+$. To ensure accuracy, single ion monitoring (SIM) mode was used to exclusively detect fucose and glucose ions.

Two methods were used to determine fucose concentrations by LC-MS. The first method, a calibration curve, related the concentrations of fucose solutions to the peak areas they produced in the LC-MS chromatogram. Fucose standards of 400 μM , 800 μM , 1200 μM , and 1600 μM were run through LC-MS, and their peak areas were plotted versus concentration, producing a calibration curve (Figure 8). Concentrations of unknown samples were found by running them through LC-MS to find a peak area for the solution, then extrapolating the concentration from the calibration curve.

The second method of quantitation utilized a similar sugar, glucose, as an internal standard. A spike of glucose was injected into a fucose standard. A response factor

Figure 8. A calibration curve was made using four standard solutions of fucose ranging from 400 μM to 1600 μM run on the LC-MS. The peak area from LC increased linearly with concentration, as the line suggests. Concentration was plotted versus peak area.

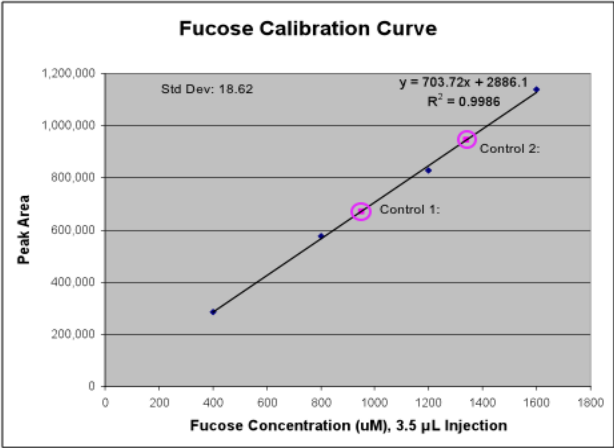


Figure 9. LC-MS chromatogram. Glucose was used as an internal standard for fucose. By spiking in a known amount of glucose, the unknown concentration of fucose could be calculated based on relative peak areas. This particular chromatogram shows an unknown concentration of fucose, which was accurately determined using the internal standard method.

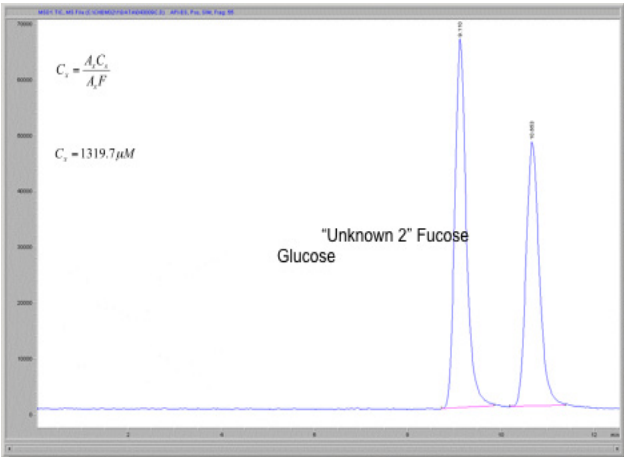
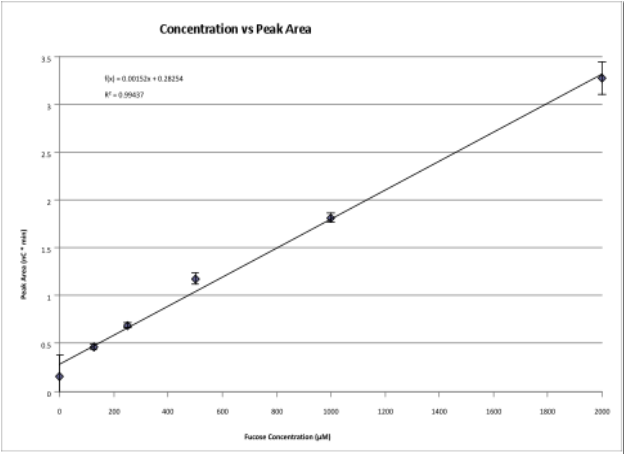


Figure 10. Fucose Calibration Curve prepared by HPLC-PAD. The Calibration curve was produced by serial dilutions of a 2000 μM solution. The curve shows strong correlation between fucose concentration and integrated detector response.



was calculated, which is a way to measure how the instrument responds to the two sugars. The response factor relates the peak areas produced by the two different sugars to their concentrations; this response factor can then be used to calculate an unknown fucose concentration when glucose has been added. Once unknown fucose samples were spiked with glucose and run through the LC-MS, the previously determined response factor was plugged into a formula to calculate the concentration of fucose (Figure 9).

RESULTS, DISCUSSION AND IMPLICATIONS

RAPID METHODS

The Yellow Card and CRS tests were performed on the “unknown” fucose solutions. The Yellow Card method produced estimates of 900 μM for unknown 1 and 1400 μM for unknown 2. The Yellow Card estimates differed by 3.5% and 6.5%, respectively, from the actual concentrations of the solutions (unknown 1 was 933 μM ; unknown 2 was 1314 μM). The CRS test produced estimates of 950 μM for unknown 1 and 1400 μM for unknown 2, differing by 1.8% and 6.5%, respectively, from the actual concentrations. These results suggest that the Yellow Card and CRS tests are sufficiently sensitive to determine whether fucose levels are normal or elevated.

INSTRUMENTAL METHODS

The fucose concentrations in the unknown samples were calculated to be 888.7 μM and 1227 μM by extrapolation from the HPLC-PAD calibration curve (Figure 10). Both of the instrumentally determined concentrations were within 7% of their actual values.

After fucose standards were run on the LC-MS, two fucose samples of unknown concentrations were

studied. The first unknown gave a fucose peak area of 671042.9, which correlated to $949.5 \pm 18.62 \mu\text{M}$ from the calibration curve and 970.84 μM from the internal standard. The actual concentration of fucose was 933 μM , yielding calibration curve and internal standard errors of 1.7% and 3.4%, respectively. The second unknown gave a fucose peak area of 945248.6, which correlated to $1339 \pm 18.62 \mu\text{M}$ from the calibration curve and 1319.7 μM from the internal standard. The actual concentration of fucose was 1314 μM , yielding calibration curve and internal standard error of 1.9% and 0.38%.

From these experimental tests, it was accepted that LC-MS was sensitive enough to quantify the levels of fucose and accurately classify them as normal, precancerous, or cancerous. These results imply that LC-MS produces sufficiently sensitive data to verify the results of the colorimetric methods.

CONCLUSIONS

The two unknown solutions were tested with each of the methods. Results indicate that LC-MS provides the most accurate quantitative results, as error was less than 3.9%. HPLC-PAD was also able to quantify unknown samples with 7% error. LC-MS is useful for quantifying fucose and it provides mass-specific information that could be beneficial for identifying compounds in a biological sample. The two rapid methods successfully identified the unknowns as normal, precancerous, or cancerous levels of fucose. The Yellow Card method is advantageous because it requires no heat and provides a lasting color solution that can easily be identified using the Yellow Card. It is promising for application in a clinical setting because it provides quick, visual results.

Successful classification of fucose levels as normal, precancerous, or

cancerous by both of the rapid methods implies that, upon further development, these methods may one day be applied to a clinical setting. For these experiments, pure fucose was analyzed to verify our proposed methods. Because these simple methods are not selective for fucose, any future experiments mimicking a biological system would also require separation of fucose and/or fucosylated glycoproteins from other sugars in blood or serum.

The results for the instrumental methods and the rapid methods agree. Although the Yellow Card method and the CRS method had high limits of detection, they are still useful methods for the early detection of possibly cancerous or precancerous conditions. Further experimentation could focus

on reducing the amounts of reactants needed as well as modifying the Yellow Card method to require less hazardous reagents.

The next step for the project involves studying model biological systems. Solutions containing both sugar and glycoproteins should be analyzed with each of the methods to more closely mimic a blood or serum sample. This would determine whether the biological matrix affects visualization or quantitation. After studies of model systems, separation of fucose from biological samples, such as blood or serum, could be applied towards a clinical setting after development of additional preparation and purification steps.

END NOTES

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